REPLICATIVE DNA SYNTHESIS IN TISSUES OF THE RAT EXPOSED TO AGED AND DILUTED SIDESTREAM SMOKE

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Male Sprague-Dawley rats were exposed to aged and diluted sidestream smoke (ADSS) from Kentucky 1R4F reference cigarettes for 6 h/day, 5 days/wk, for a 13-wk period. Exposure concentrations were 0, 0.1, 1, and 10 mg ADSS/m³. Exposures were conducted in whole-body inhalation chambers. Rats were held in nose-only exposure tubes for the 6-h exposures to minimize pelt deposition and subsequent ingestion of ADSS. Groups of 10 rats from each exposure group were killed after 5, 28, and 90 d of exposure to examine the rates of replicative DNA synthesis; 6 rats from each exposure group were kept for a 90-day recovery period after termination of exposures to examine replicative DNA synthesis rates. Three days prior to each scheduled necropsy, an osmotic pump containing 5-bromo-2'-deoxyuridine (BrdU) was implanted subcutaneously. After necropsy, tissues were processed for examination of BrdU-containing cells at several sites. Incorporation of BrdU was assessed either by counting the number of labeled cells along a length of an epithelial surface or by counting the number of labeled cells in an area of tissue. Tissues examined were from the nasal cavity, ventral larynx, and trachea, in addition to bronchial, bronchiolar, and alveolar regions of the lung. Endocardium, myocardium, epicardium, and aortic smooth muscle sites were also examined. Increased replicative DNA synthesis occurred in some sites of the respiratory tract at the 5-day timepoint at the mid or high exposure concentrations, although at 28 and 90 days, these effects had diminished in intensity or were not present, indicating adaptation to the ADSS exposure. The only tissues with elevated rates of replicative DNA synthesis at 90 days were the cuboidal and respiratory epithelium at the most rostral portion of the nasal cavity at the highest exposure concentration. Increased rates of replicative DNA synthesis were not noted in heart tissues or lung alveolar epithelium at any concentration at any time point. Examination of rats killed after the end of the 90-day recovery period indicated that the increase in replicative DNA synthesis was not sustained after termination of exposures. The no observed effect level (NOEL) for increased replicative DNA synthesis after subchronic exposure to ADSS in the rat is greater than 1 mg ADSS/m³.

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Incorporation of a thymidine analogue, 5-bromo-2'-deoxyuridine (BrdU), into DNA during replicative DNA synthesis is a frequently used method for estimating cell proliferation (Doolittle et al., 1992). Detection of increased replicative DNA synthesis has been advanced as a component of rodent subchronic and chronic toxicology studies that provides information about the cell proliferative potential of test compounds in the rodent (Butterworth, 1991; Clayson et al., 1989; Cohen & Ellwein, 1990).

Aged and diluted sidestream cigarette smoke (ADSS) is a complex mixture that contains both genotoxic and nongenotoxic compounds (Guerin et al., 1992). Limited experimental data are available concerning the potential cell proliferative activity of ADSS. The available experimental data from inhalation of ADSS includes histopathological evaluation after a 90-day study period using 10-h exposures to a single elevated concentration of ADSS (von Meyerinck et al., 1989) and 14- and 90-day studies using 6-h exposures at 3 exposure concentrations (Coggins et al., 1992, 1993). During the conduct of the 90-day inhalation study using 3 concentrations of ADSS, there were no treatment-related clinical signs or reduction in body weight compared to a sham exposed group. At the termination of the 90-day study, the only histological change observed in the rats was a mild hyperplastic response in the most rostral portion of the nasal cavity, found only in animals exposed to the 10 mg/m^3 concentration (Coggins et al., 1993). To gain a better appreciation of the mild hyperplastic changes in the rat nose, experimental determination of replicative DNA synthesis was accomplished in groups of rats exposed simultaneously with those that were examined for histological changes in the previously reported study.

In the present evaluation, an increase in cell labeling in the nasal cuboidal epithelium in rats killed at the end of the 90-day study coincided with the histologic changes reported previously. No changes in replicative DNA synthesis rates were noted in rats killed after subchronic exposure to ADSS at concentrations of 0.1 or 1 mg/m^3. Examination of rats killed after a 90-day recovery period without further exposure to ADSS revealed that the increase in replicative DNA synthesis rate was not sustained after 13 wk of exposure to HEPA-filtered and humidified room air.

MATERIALS AND METHODS

Detailed descriptions of study design, ADSS exposure, animal husbandry, and histopathology results have been previously published (Ayres et al., 1994; Coggins et al., 1993).

Inhalation Exposure

Rats were exposed to ADSS in 2.3-m^3 whole-body exposure chambers at target concentrations of 0, 0.1, 1.0, or 10 mg/m^3 for 6 h/day, 5 days/wk, for a 13-wk period. These exposure concentrations were established at factors of approximately 1, 10, and 100 times higher than the airborne particulate concentration of 120 μg/m^3, detected in areas where smoking was permitted, of

which only about half was attributed to tobacco smoke (Guerin et al., 1992). Rats were held in nose-only exposure tubes during 6-h exposures to the ADSS exposure environment to minimize pelt deposition and subsequent ingestion of ADSS. Groups of rats were killed after 5, 28, or 90 days of exposure. Additional rats were killed after a 13-wk recovery period during which rats were not further exposed to ADSS.

Experimental Animals

Male Sprague-Dawley rats approximately 8 wk of age at the initiation of inhalation exposures were used for this study. Ten rats in each of the 4 exposure groups were removed from the study and examined for replicative DNA synthesis rates after 5, 28, or 90 days of exposure. Six rats in each of the exposure groups were kept for an additional 13-wk recovery period after termination of inhalation exposures and then used for quantification of replicative DNA synthesis.

The rats were housed and cared for in accordance with the Animal Welfare Act of 1970 and amendments (Public Law 91-579), as set forth in CFR Title 9, Part 3 Sub-part E. Animal care procedures followed those contained within the DHHS document Guide for the Care and Use of Laboratory Animals (NIH publication 86-23). Serological analyses confirmed that the rats used were free of infectious disease. Rats were provided ad libitum access to Purina 5002 Rodent chow and drinking water except during inhalation exposures. Animal use for this study was reviewed and approved by an Institutional Animal Care and Use Committee.

5-Bromo-2'-deoxyuridine (BrdU) Administration

BrdU (Sigma Chemical Company, St. Louis, MO) at a concentration of 20 mg/ml was contained within surgically implanted Alzet osmotic pumps (Alza Corporation, Palo Alto, CA) designed to deliver 10 μl/h for at least 3 days. In preparation for the pump implant, rats were anesthetized with 70% carbon dioxide (CO_2) in air. The inhalant CO_2 was used because of its reduced potential to cause lasting side-effects after brief periods of anesthesia (Lumb & Jones, 1984). The surgical site was first clipped to remove hair and then swabbed with a dilute Betadine (Purdue Frederick, Norwalk, CT) solution. An incision was made in the skin approximately 2.5 cm in length. The osmotic pump was then implanted subcutaneously over the dorsal thoraco-lumbar region and the incision was closed with Auto Wound Clips (Becton-Dickinson, Mountain View, CA) using a surgical stapler (Biomedical Instruments, Rockville, MD). The osmotic pump was implanted 72 h prior to the scheduled necropsy. All rats were routinely monitored for clinical signs of infection until the scheduled necropsy.

Collection of Tissues

Rats were anesthetized with 70% CO_2 in air and while under anesthesia were exsanguinated. Tissues selected for examination were those that had

previously been shown to demonstrate histologic effects at the highest exposure concentration (nasal tissues) (Coggins et al., 1993), other portions of the respiratory system so that a complete evaluation of the organs associated with the route of exposure could be accomplished, and heart to examine potential cardiovascular effects. At necropsy, tissues were removed from each animal and fixed in 10% neutral buffered formalin (NBF) at a volume dilution of 1 part tissue to at least 15 parts formalin (Feldman & Seely, 1988). Lungs were distended with NBF at a pressure of approximately 25 cm water. The trachea was ligated after distension of the lung. The nasal cavity was perfused with NBF by flowing fixative through the nasal pharynx to assure proper fixation of the nasal epithelium.

The following tissues were collected for examination of BrdU incorporation: nasal cavity, larynx, proximal end of the trachea, lung mainstem bronchus, lung, heart, and duodenum. The nasal cavity was cut into four regions defined as section 1, 2, 3, and 4 according to the method of Young (1981). Figure 1 shows a schematic representation of the nasal cavity in cross section at section 1. Cuboidal, respiratory, and squamous epithelia were examined in nasal section 1. Cuboidal, respiratory, and two areas of olfactory epithelia were examined in nasal section 2. Two regions of olfactory epithelium and 1 region of respiratory epithelium were examined in nasal section 3. Ventral laryngeal and squamous epithelial regions were prepared according to the methods of Sagartz et al. (1992). Lung bronchial, bronchiolar, and alveolar regions were examined as individual sites. Endocardium, myocardium, epicardium, and aortic smooth muscle sites were also examined.

Tissue Processing and Immunochemical Staining

Soft tissues were fixed in NBF for 24 h and then dehydrated in an ascending alcohol series from 70 to 100% ethanol and placed in xylene for

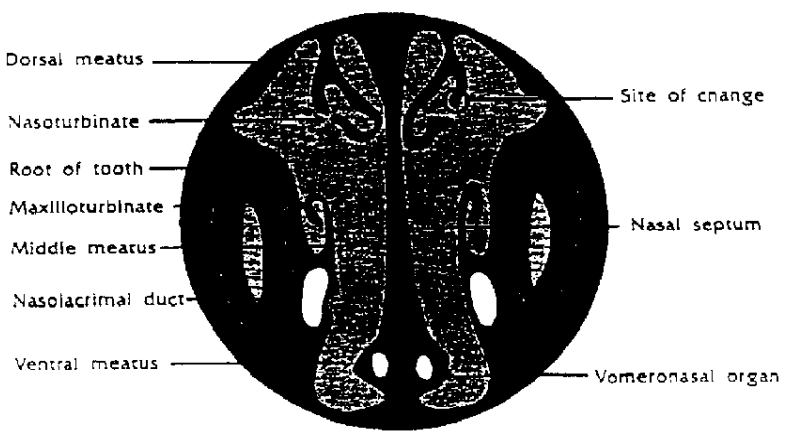


FIGURE 1. Drawing of a cross section of the rostral nasal cavity demonstrating the site of change in the nasal cuboidal epithelium of the turbinate.

infiltration and embedding in paraffin. After fixation, the nasal cavity was placed in 5% formic acid with an ion-exchange resin for decalcification for 5 days prior to dehydration, infiltration, and embedding in paraffin. Sections of tissue embedded in paraffin blocks were cut at 5 μm thickness using a conventional rotary microtome. A cross section of duodenum from each animal was included on each tissue slide prepared to gather qualitative information about the efficiency of osmotic pump delivery of BrdU and immunostaining. Since very few sections were deemed to be insufficiently stained, no attempt was made to recut and restain slides that were not appropriately stained.

Sections of tissue on glass microscope slides were treated in the following manner in preparation for immunostaining. Slides were placed in two successive 5-min solvent baths of xylene to remove paraffin. Slides were then placed in two 5-min solvent hydration baths of ethyl alcohol at 100, 95, and 70% in water. After the 70% ethanol bath, slides were rinsed twice in phosphate-buffered saline (PBS) for 5 min.

Slides were placed in 1 *N* hydrochloric acid for 60 min at 37°C to partially denature DNA. The hydrochloric acid was neutralized with two 5-min rinses in PBS. A 0.05% protease treatment (protease type 24, Sigma Chemical Company, St. Louis, MO) for 10 min at 27°C was used to increase the accessibility of BrdU binding sites. Endogenous alkaline phosphatase was blocked with application of 5 m*M* Levamisole (Vector Laboratories, Burlingame, CA) for 10 min. Slides were then washed 2 times, for 5 min in each wash, with PBS. Nonspecific binding sites were blocked with normal horse serum (Vector Laboratories, Burlingame, CA) for 20 min at room temperature. The horse serum was not rinsed from the slide before application of the BrdU antibody.

Immunostaining for the presence of BrdU incorporated into DNA used Alkaline Phosphatase Supersensitive reagents (Biogenics, San Ramon, CA). The BrdU primary antibody was applied at a dilution of 1 : 100 for the nasal sections. All other sections used a 1 : 200 dilution. The primary antibody was allowed to react overnight at 4°C. The antibody was removed with two 5-min rinses in PBS. A 1 : 10 dilution of Supersensitive LINK (Biogenics, San Ramon, CA) was then applied to the sections for 30 min at room temperature. Slides were then rinsed twice for 5 min in PBS. A 1 : 10 dilution of Supersensitive LABEL (Biogenics, San Ramon, CA) was then applied to the sections for 20 min followed by two 5-min rinses in PBS. Supersensitive CHROMOGEN (Biogenics, San Ramon, CA) was then applied to the sections for 10 min. Negative control slides were included when appropriate in the immunostaining procedure. Sections were then rinsed in distilled water and allowed to dry. Sections were counterstained with Mayer's hematoxylin as needed to visualize the non-stained tissues. Sections were covered with Crystal Mount (Biomeda Corp., Foster City, CA) prior to scoring labeled cells.

Scoring of BrdU Labeled Cells

Regions of interest, as defined next, were initially scored with knowledge of the treatment group. Respiratory tract epithelia, except where noted, were

scored on a unit length labeling index basis (Monticello et al., 1989). As described by Monticello et al. (1989), the length of nasal section 1 cuboidal epithelium of the dorsal nasal concha encompasses 4–7 mm. In this study, a more conservative approach was used whereby only the tip of the dorsal nasal concha was evaluated because it previously had been identified as a localized site that was responsive to the effects of the high exposure concentration of ADSS (Coggins et al., 1993). Scoring of cells only at the tip of the dorsal nasal concha maximized detection of effects by exclusion of cells from a nonsensitive region. Right ventricle heart epicardium and endocardium were also scored using unit length labeling index. Heart myocardium and aortic smooth muscle were scored as the number of labeled cells in a 20× field of view on a light microscope. A qualitative assessment of the technical quality of immunostaining was made on the duodenum cross section on each slide by the pathologist. If the overall technical quality of BrdU immunostaining was deemed unsatisfactory or the tissue section was not representative of the region of interest because of artifacts, the tissue site was not scored.

Description of Sites Scored

The regional location and unit length of the sites scored for number of cells that incorporated BrdU are described next. Several sites were evaluated as labeled cells per area as indicated.

Nasal Section 1 A 1.5-mm total length of cuboidal (transitional) epithelial tissue was evaluated for the number of cells that incorporated BrdU. Three separate 0.5-mm sites at the tip and adjacent areas of the dorsal nasal concha were counted and combined for a total count of 1.5 mm. The respiratory epithelium was represented by counting 1 mm length along the middle portion of nasal septum. A 1-mm length of the squamous epithelium of the ventral region of the meatus was scored.

Nasal Section 2 A total of 1.5 mm of the length of the cuboidal (transitional) epithelium of the dorsal nasal turbinate was counted in the same manner as nasal section 1. Epithelial cells in 1 mm of respiratory epithelium were counted in a region of the septum near the ventral portion, and 1 mm of olfactory epithelium was evaluated in the dorsal region of both sides of the nasal cavity (two sides counted separately). During microtomy, no attempt was made to ascertain left or right side of the nasal cavity. Thus, even though both sides of the nasal cavity olfactory epithelium were counted separately, the left or right side orientation for olfactory site 1 and site 2 was not determined.

Nasal Section 3 The respiratory epithelium (1 mm) of nasal section 3 was evaluated in the ventral portion of the nasal cavity. Olfactory epithelium (1 mm) was counted on both sides of the nasal septum at the extreme dorsal region of the septum (two sides considered separately). As in nasal section 2, the left and right side orientation for olfactory site 1 and site 2 was not determined.

Larynx A 1-mm length of the pseudostratified epithelium of the ventral larynx overlying the seromucinous glands as described by Sagartz et al. (1992) was evaluated. A second site, from the laryngeal squamous epithelium (1 mm) along the lateral sides of the larynx, was scored as a separate site.

Trachea A total of 1.5 mm of respiratory epithelium lining cross sections of the trachea was evaluated in 3 separate 0.5-mm sites. The three sites were combined together to total 1.5 mm.

Lung The left lobe of the lung was used for evaluation of cells that had incorporated BrdU, and 1 mm of length from a longitudinal section of the bronchial epithelium along the mainstem bronchus and 1 mm of bronchiolar epithelium lining the terminal bronchiole were evaluated. Labeled cells, without distinction of a particular cell type, were counted in a 0.25-mm^2 area of alveolar tissue.

Heart A length of 1 mm of the endocardium and epicardium was evaluated separately from the myocardium. The number of labeled cells present in the myocardium was scored as number per 0.25-mm^2 area. The number of labeled cells in a longitudinal section of aortic smooth muscle was scored in a similar manner.

Statistical Analysis

Cell count data were initially evaluated for conformance to a parametric data analysis approach. Both normality of distribution and homogeneity of variance were evaluated. Bartlett's test and Levine's test for homogeneity of variance were employed. The Wilks-Shapiro test for evaluation of normally distributed data was employed. Most of the cell count data could be not analyzed by a parametric statistical approach because of a lack of normality and heterogeneous variance. A square-root transformation was applied to the cell count data in an attempt to reduce the heterogeneity of variance. Because most of the data sets still exhibited heterogenous variance, a non-parametric statistical test, the Kruskal-Wallis rank sum test, was used at a .05 level of significance. No mathematical method to eliminate potential outliers was employed.

Statistical Comparisons

Responses in sham-exposed rats were compared between the 5 and 90, and between the 90 and 180 day time points. Statistical analysis of the sham group revealed a general decline in BrdU incorporation with increasing age of the animal on study. Therefore, statistical comparisons were limited to cell count responses between sham and ADSS-exposed treatment groups within a time point to avoid the confounding effect of age of the rat or time on study.

RESULTS

Effect of Study Day

Analysis of the number of cells, in animals of the sham group, that had incorporated BrdU demonstrated that statistically significant differences were

found within the same tissue site when compared between the initial sampling period (day 5) and the termination of inhalation exposures (day 90), and between the termination of inhalation exposures (day 90) and the end of the recovery period (day 180). Generally, there was a decline in cell labeling from day 5 to day 90, with a somewhat smaller decline noticed between day 90 and day 180. In the upper airways of the sham-exposed group, cell labeling declined about 50% between day 5 and 90 and then was found to be similar at 90 and 180 days. In the lung epithelium of sham-exposed rats, cell labeling displayed a greater degree of variability, with the greatest decline in cell labeling seen in the conducting airways of the bronchus.

Exposure-Related Statistically Significant Findings

Statistically significant changes were observed in several tissues after exposure to ADSS at concentrations ranging from 0.1 to 10 mg/m^3. A complete list of number of labeled cells and statistical findings is noted in Tables 1–4.

Day 5

Statistically significant differences were noted in several tissues when ADSS exposed animals were compared to sham exposed animals at day 5. In nasal section 1 of cuboidal epithelium, cell labeling was increased significantly in both the mid and high exposure groups (Figure 2a). At the mid exposure concentration of 1.0 mg ADSS/m^3, cell labeling was approximately double that of controls, whereas at the high exposure concentration of 10 mg ADSS/m^3, cell labeling was approximately fourfold higher than sham-exposed controls.

Cell labeling was significantly decreased in the squamous epithelium of the nasal section 1 at the high exposure concentration (Figure 2b).

Incorporation of BrdU was significantly increased in the respiratory epithelium of nasal section 2 at the high exposure concentration where cell labeling was increased less than twofold (Figure 2d).

Cell labeling in the olfactory site 1 of nasal section 2 was significantly decreased in the high exposure group when compared to the sham-exposed group (Figure 2e). At the other side of the nasal cavity at the olfactory site 2 of nasal section 2, cell labeling was significantly decreased in the low exposure group when compared to the sham exposed group (Figure 2f).

In the middle airways, no significant differences were noted.

In the lung bronchiolar epithelium, an approximate twofold increase in cell labeling in the high exposure group was significantly different from the sham-exposed group (Figure 2g).

Day 28

Only two tissue sites from ADSS-exposed animals displayed statistically significant findings when compared to the sham-exposed animals at day 28.

TABLE 1. Number of BrdU-Labeled Cells in Tissues Examined after 5 Days of Exposure to Aged and Diluted Sidestream Smoke at 0 (sham), 0.1 (low), 1.0 (mid), and 10 (high) mg/m³

Tissue	Length or area counted	Exposure group: Sham Mean	Sham SEM	Sham n	0.1 mg ADSS/m³ Mean	0.1 mg ADSS/m³ SEM	0.1 mg ADSS/m³ n	1.0 mg ADSS/m³ Mean	1.0 mg ADSS/m³ SEM	1.0 mg ADSS/m³ n	10 mg ADSS/m³ Mean	10 mg ADSS/m³ SEM	10 mg ADSS/m³ n
Nasal 1 cuboidal	1.5 mm	40.4	3.4	9	51.5	2.7	10	88.5[a]	20.3	10	168.1[a]	21.5	10
Nasal 1 respiratory	1 mm	22.3	1.3	9	23.8	1.6	10	26.1	1.3	10	23.7	3.1	10
Nasal 1 squamous	1 mm	291.9	5.8	9	297.4	7.5	10	299.5	10.1	10	259[a]	5.8	10
Nasal 2 cuboidal	1.5 mm	37.2	2.7	9	43.4	5.6	10	35.7	2.9	10	31.2	3.2	10
Nasal 2 respiratory	1 mm	28.3	3.2	9	29.1	3.3	10	29.8	2.8	10	48[a]	4.4	10
Nasal 2 olfactory 1	1 mm	29.1	5.6	9	13.8	4.0	10	27.1	5.0	10	6[a]	1.8	10
Nasal 2 olfactory 2	1 mm	31.2	6.3	9	10.7[a]	3.6	10	23.8	5.4	10	18.9	6.1	10
Nasal 3 respiratory	1 mm	19.2	2.6	9	20.3	2.5	9	19.2	2.1	10	23.9	4.3	10
Nasal 3 olfactory 1	1 mm	19.6	7.5	8	5.7	2.6	10	12.4	5.5	10	6.6	1.3	10
Nasal 3 olfactory 2	1 mm	13.6	5.3	8	2.9	0.6	10	7.2	2.5	10	8.4	2.2	10
Larynx ventral epithelium	1 mm	5.1	1.5	7	6	0.7	10	6.7	0.7	10	13	3.5	10
Larynx squamous epithelium	1 mm	13.6	3.3	7	8.6	0.9	10	12.6	2.5	10	13.3	3.2	10
Larynx/trachea	1.5 mm	13.9	1.3	9	9.6	0.9	10	16.7	2.1	10	12	2.6	10
Lung, bronchial	1 mm	8.3	1.6	9	4.9	0.6	10	7.1	1.1	10	14	2.7	10
Lung, bronchiolar	1 mm	5.3	1.5	9	4	0.6	10	6.8	0.8	10	12.2[a]	2.2	10
Lung, alveolar	0.25 mm²	6.9	0.9	9	4	0.5	10	6.3	0.9	10	9.9	1.8	10
Heart, endocardium	1 mm	0.3	0.2	9	0.1	0.1	10	0	0.0	10	0.4	0.2	10
Heart, epicardium	1 mm	0.3	0.2	9	0.5	0.2	10	0.6	0.2	10	0.3	0.2	10
Heart, myocardium	0.25 mm²	1.6	0.4	9	1.7	0.4	10	1.8	0.4	10	2.2	0.4	10
Aortic smooth muscle	0.25 mm²	4	0.4	9	3.1	0.5	10	3.3	0.5	10	3	0.4	10

[a]Significantly different from sham at $p \leq .05$.

TABLE 2. Number of BrdU-Labeled Cells in Tissues Examined at Study Day 28 after Exposure to Aged and Diluted Sidestream Smoke at 0 (sham), 0.1 (low), 1.0 (mid), and 10 (high) mg/m³

Tissue	Length or area counted	Exposure group: Sham Mean	Sham SEM	Sham n	0.1 mg ADSS/m³ Mean	0.1 mg ADSS/m³ SEM	0.1 mg ADSS/m³ n	1.0 mg ADSS/m³ Mean	1.0 mg ADSS/m³ SEM	1.0 mg ADSS/m³ n	10 mg ADSS/m³ Mean	10 mg ADSS/m³ SEM	10 mg ADSS/m³ n
Nasal 1 cuboidal	1.5 mm	31.9	3.2	10	36.5	2.8	10	35.2	2.4	10	169.7[a]	17.1	10
Nasal 1 respiratory	1 mm	14.2	1.1	10	18.5	1.0	10	15.2	1.2	10	27.5[a]	4.4	10
Nasal 1 squamous	1 mm	319.4	12.9	10	351.5	11.4	10	360.9	15.2	10	371.2	12.3	10
Nasal 2 cuboidal	1.5 mm	27.8	2.3	10	28.2	1.4	10	24.3	2.5	10	26.9	3.0	10
Nasal 2 respiratory	1 mm	17.3	1.8	10	18.8	2.8	10	18.6	1.9	10	24.7	2.2	10
Nasal 2 olfactory 1	1 mm	7.5	2.2	10	6.6	3.9	10	3	2.3	10	7.6	4.0	10
Nasal 2 olfactory 2	1 mm	6.9	2.2	10	7.7	3.8	10	2.4	1.1	10	8.5	4.2	10
Nasal 3 respiratory	1 mm	21.3	2.3	9	18.2	1.5	10	19.8	1.4	10	21	4.0	10
Nasal 3 olfactory 1	1 mm	2.2	1.3	9	0.4	0.2	10	0.8	0.3	10	0.4	0.3	10
Nasal 3 olfactory 2	1 mm	2.4	1.1	9	0.9	0.6	10	1.1	0.4	10	1.3	0.3	10
Larynx ventral epithelium	1 mm	16.6	3.5	10	9.4	1.3	8	16	3.0	8	11.3	1.2	8
Larynx squamous epithelium	1 mm	39	8.8	10	26.6	6.2	8	48.6	6.8	8	39.8	8.8	8
Larynx/trachea	1.5 mm	11.3	1.5	10	8.8	0.9	10	9.8	1.0	10	8.6	1.2	10
Lung, bronchial	1 mm	5.9	1.0	10	5	0.4	10	4.7	0.5	10	5.2	0.9	10
Lung, bronchiolar	1 mm	4.9	0.9	10	5	1.1	10	3.8	0.3	10	3.5	0.5	10
Lung, alveolar	0.25 mm²	6.3	1.2	10	4.8	0.6	10	4.5	0.6	10	6.3	1.1	10
Heart, endocardium	1 mm	0.3	0.2	10	0.2	0.1	10	0.4	0.2	10	0.3	0.2	10
Heart, epicardium	1 mm	0.3	0.2	10	0.1	0.1	10	0.2	0.1	10	0.3	0.2	10
Heart, myocardium	0.25 mm²	3.5	0.7	10	2.5	0.5	10	2.3	0.3	10	1.8	0.3	10
Aortic smooth muscle	0.25 mm²	1.7	0.4	10	1.3	0.3	10	0.9	0.2	10	1.4	0.5	10

[a]Significantly different from sham at $p \leq .05$.

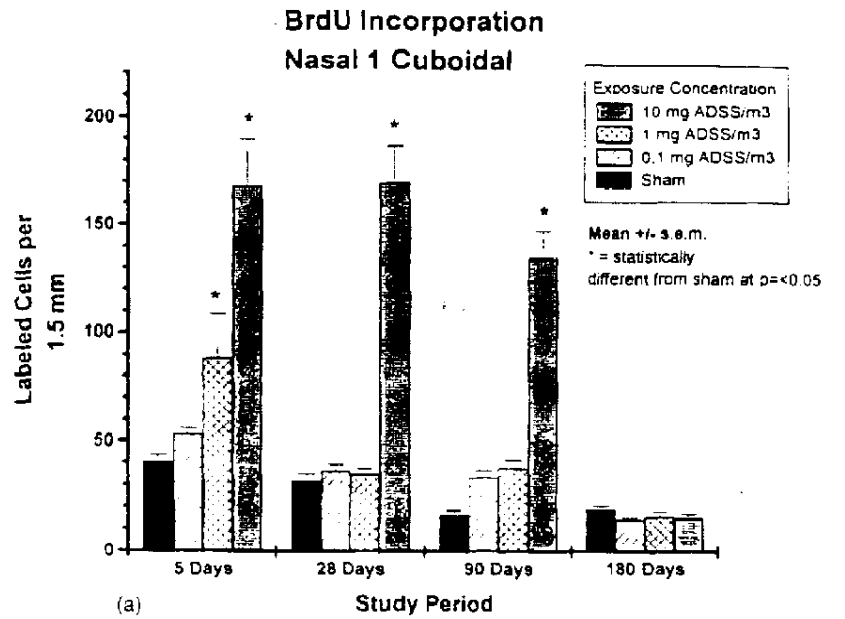
TABLE 3. Number of BrdU-Labeled Cells in Tissues Examined at the Termination of the 90-Day Subchronic Study of Aged and Diluted Sidestream Smoke at 0 (sham), 0.1 (low), 1.0 (mid), and 10 (high) mg/m³

Tissue	Length or area counted	Exposure group: Sham			0.1 mg ADSS/m³			1.0 mg ADSS/m³			10 mg ADSS/m³		
		Mean	SEM	*n*	Mean	SEM	*n*	Mean	SEM	*n*	Mean	SEM	*n*
Nasal 1 cuboidal (initial score)	1.5 mm	16.4	1.9	10	33.3[a]	3.2	10	37.7[a]	3.5	10	134.9[a]	12.0	9
Nasal 1 cuboidal (blind reread)	1.5 mm	18.7	1.7	10	28.6	3.0	10	28.2	3.3	10	121.4[a]	14.2	9
Nasal 1 respiratory	1 mm	9.7	2.1	10	14.8	1.9	10	13.2	1.4	10	20[a]	2.6	10
Nasal 1 squamous	1 mm	331.9	10.3	10	340.8	10.2	10	323.3	11.5	10	342.3	8.1	10
Nasal 2 cuboidal	1.5 mm	24.5	2.4	10	22.6	2.7	10	20.2	1.0	10	23	1.7	10
Nasal 2 respiratory	1 mm	17.2	2.9	10	13	1.4	10	14.9	1.6	10	17.6	1.5	10
Nasal 2 olfactory 1	1 mm	11.5	4.4	10	6	2.2	10	13.4	4.1	9	10.7	7.7	10
Nasal 2 olfactory 2	1 mm	11.9	4.7	10	4.9	2.5	10	9.1	3.1	9	9.4	6.1	10
Nasal 3 respiratory	1 mm	14.8	2.2	10	13.5	1.3	10	14.3	1.3	10	19.4	2.1	10
Nasal 3 olfactory 1	1 mm	1.2	0.6	10	1.5	0.7	10	2.9	1.4	10	0.7	0.3	10
Nasal 3 olfactory 2	1 mm	4.8	3.0	10	1.7	0.9	10	6	3.6	10	0.4	0.3	10
Larynx ventral epithelium	1 mm	13.2	4.1	10	13.8	6.4	6	18.9	5.2	9	29.3	6.8	9
Larynx squamous epithelium	1 mm	55.9	19.0	10	21.7	6.5	6	28.2	7.2	9	20.4	5.3	9
Larynx/trachea	1.5 mm	6.6	0.8	10	6.3	0.7	10	4.5	0.7	10	7.3	1.1	10
Lung, bronchial	1 mm	3.9	0.5	10	3.6	0.4	10	5.3	0.7	10	5.1	0.8	10
Lung, bronchiolar	1 mm	5.3	0.9	10	3.8	0.5	10	3.9	0.7	10	5.3	1.2	10
Lung, alveolar	0.25 mm²	8.7	1.2	10	6.3	0.4	10	6.3	0.7	10	9.3	0.9	10
Heart, endocardium	1 mm	0.5	0.2	10	0.4	0.2	10	0.5	0.3	10	0.3	0.2	10
Heart, epicardium	1 mm	0.2	0.1	10	0.5	0.3	10	0.3	0.2	10	0.5	0.3	10
Heart, myocardium	0.25 mm²	3.9	0.6	10	2.4	0.5	10	3.8	0.9	10	3.2	0.7	10
Aortic smooth muscle	0.25 mm²	2.5	0.8	10	1	0.3	10	1.7	0.4	10	1.5	0.5	10

[a]Significantly different from sham at $p \leq .05$.

TABLE 4. Number of BrdU-Labeled Cells in Tissues Examined at Study Day 180 after a 90-Day Recovery Period Without Smoke Exposure

Tissue	Length or area counted	Exposure group: Sham Mean	Sham SEM	Sham n	0.1 mg ADSS/m³ Mean	0.1 mg ADSS/m³ SEM	0.1 mg ADSS/m³ n	1.0 mg ADSS/m³ Mean	1.0 mg ADSS/m³ SEM	1.0 mg ADSS/m³ n	10 mg ADSS/m³ Mean	10 mg ADSS/m³ SEM	10 mg ADSS/m³ n
Nasal 1 cuboidal	1.5 mm	18.8	1.6	6	14.5	0.7	6	16	2.1	6	15.2	1.8	5
Nasal 1 respiratory	1 mm	8.3	0.9	6	5.5	0.8	6	5.7	0.9	6	5.2	1.4	5
Nasal 1 squamous	1 mm	341.5	15.8	6	364.5	12.1	6	387.3	19.8	6	385.2	11.4	5
Nasal 2 cuboidal	1.5 mm	11.3	1.8	6	10.7	0.8	6	13.2	2.3	6	15.4	6.5	5
Nasal 2 respiratory	1 mm	8.3	1.1	6	8.3	1.5	6	9.3	2.1	6	10.8	2.3	5
Nasal 2 olfactory 1	1 mm	18.5	10.4	6	15.5	4.2	6	12.7	10.0	6	6.4	3.3	5
Nasal 2 olfactory 2	1 mm	23	12.1	6	11.5	3.7	6	8.7	6.3	6	9	4.7	5
Nasal 3 respiratory	1 mm	13.7	3.4	6	13.8	1.8	6	9	1.0	6	13	3.0	5
Nasal 3 olfactory 1	1 mm	6.8	3.9	6	6.5	4.4	6	0.3	0.2	6	13	8.5	5
Nasal 3 olfactory 2	1 mm	3.3	2.4	6	7.2	3.6	6	0.7	0.5	6	7.2	5.3	5
Larynx ventral epithelium	1 mm	10.8	4.8	4	13.8	3.1	6	12.5	2.5	6	16	5.6	4
Larynx squamous epithelium	1 mm	34.5	15.5	4	14.8	4.6	6	24.5	6.1	6	21.5	8.8	4
Larynx/trachea	1.5 mm	3.7	0.5	6	3.7	0.6	6	2.5	0.2	6	3.4	0.5	5
Lung, bronchial	1 mm	3.5	1.5	6	2.7	0.6	6	1.7	0.4	6	1.8	0.2	5
Lung, bronchiolar	1 mm	2.8	0.8	6	2.7	1.0	6	3.7	1.0	6	1.6	0.6	5
Lung, alveolar	0.25 mm²	3.5	0.8	6	2.5	0.2	6	3	0.6	6	3	0.6	5
Heart, endocardium	1 mm	0.7	0.5	6	0.5	0.3	6	1.2	0.5	6	0.2	0.2	5
Heart, epicardium	1 mm	0.2	0.2	6	0.3	0.2	6	0.2	0.2	6	0.6	0.2	5
Heart, myocardium	0.25 mm²	3.3	0.6	6	3.3	0.6	6	2.7	0.4	6	3.8	0.8	5
Aortic smooth muscle	0.25 mm²	4	1.2	6	1.5	0.7	6	3	1.0	6	1.2	0.5	5



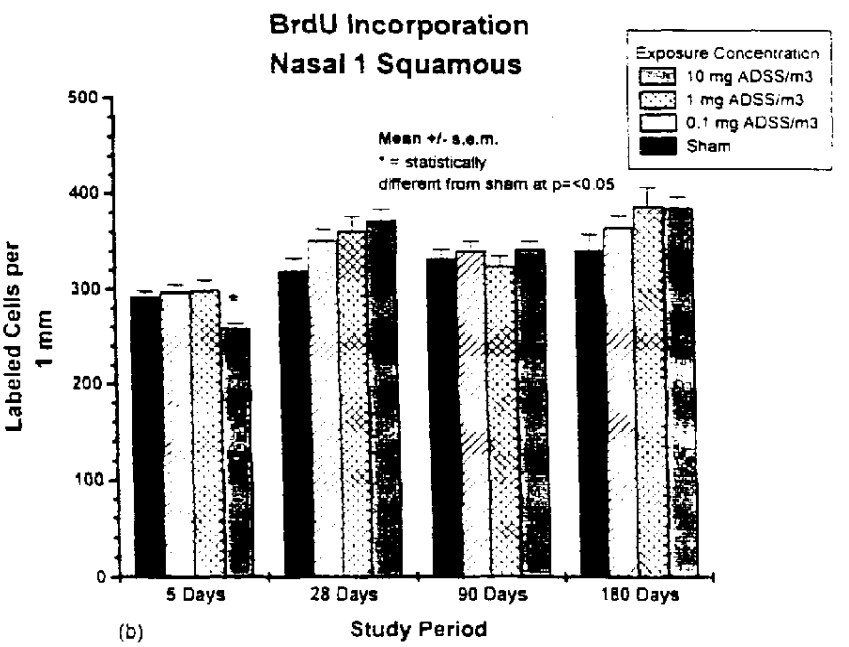
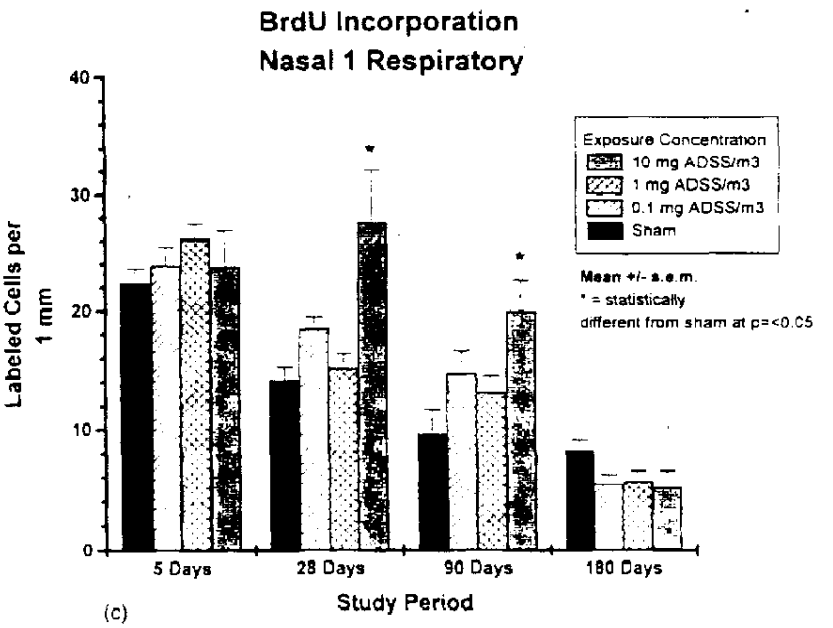
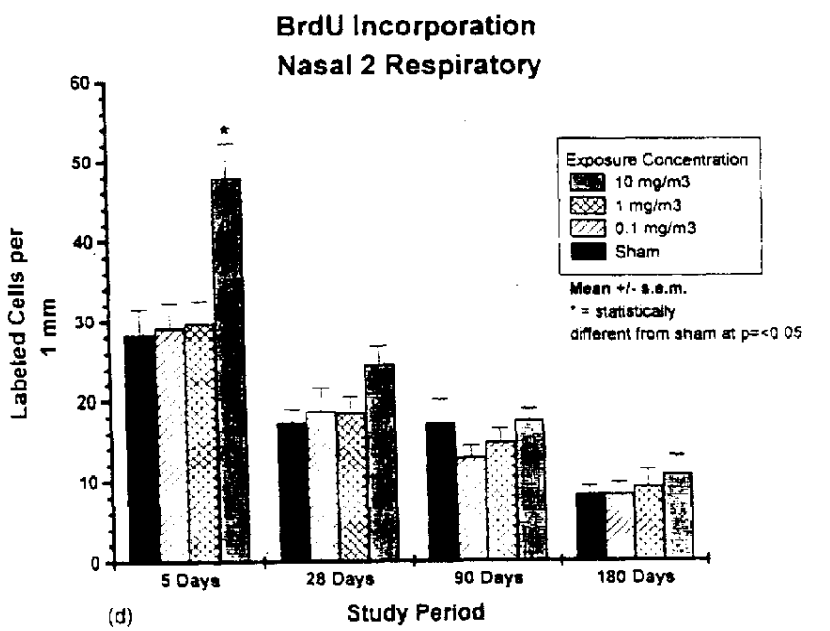


FIGURE 2. Bar charts of the number of BrdU-labeled cells at study days 5, 28, 90, and 180: (a) in the nasal section 1 cuboidal epithelium; (b) in the nasal section 1 squamous epithelium.

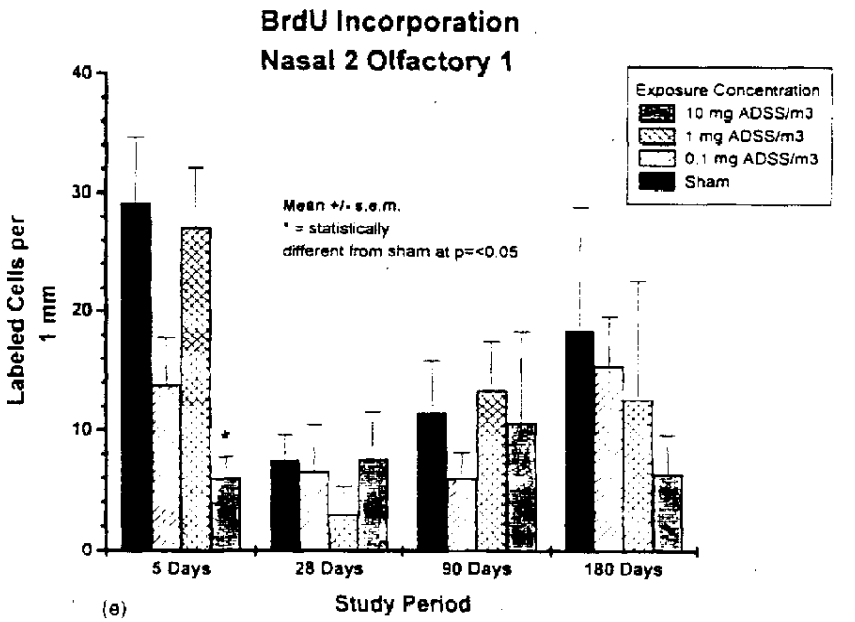


(c)



(d)

FIGURE 2. (*Continued*) Bar charts of the number of BrdU-labeled cells at study days 5, 28, 90, and 180: (c) in the nasal section 1 respiratory epithelium; (d) in the nasal section 2 respiratory epithelium.



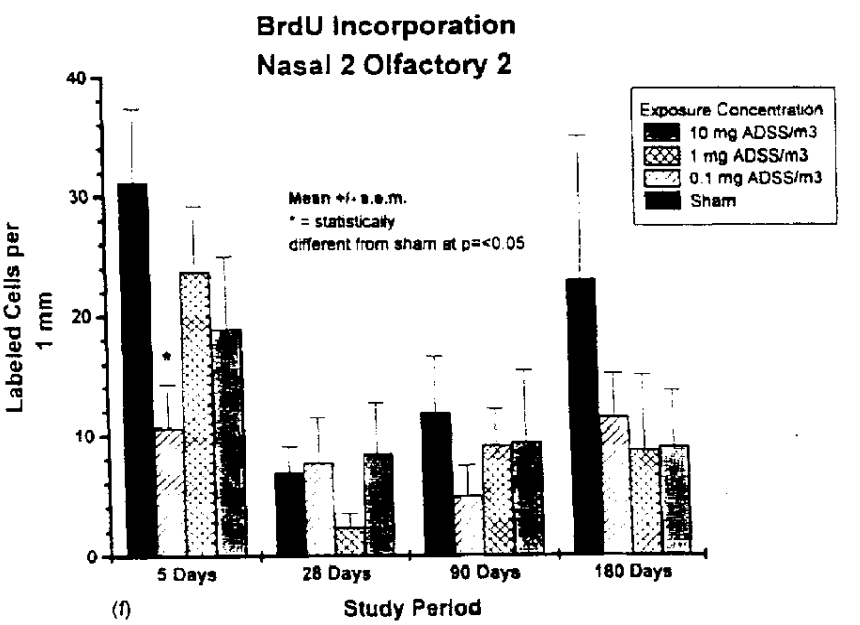


FIGURE 2. (*Continued*) Bar charts of the number of BrdU-labeled cells at study days 5, 28, 90, and 180: (e) in the nasal section 2 olfactory region 1 epithelium; (f) in the nasal section 2 olfactory region 2 epithelium.

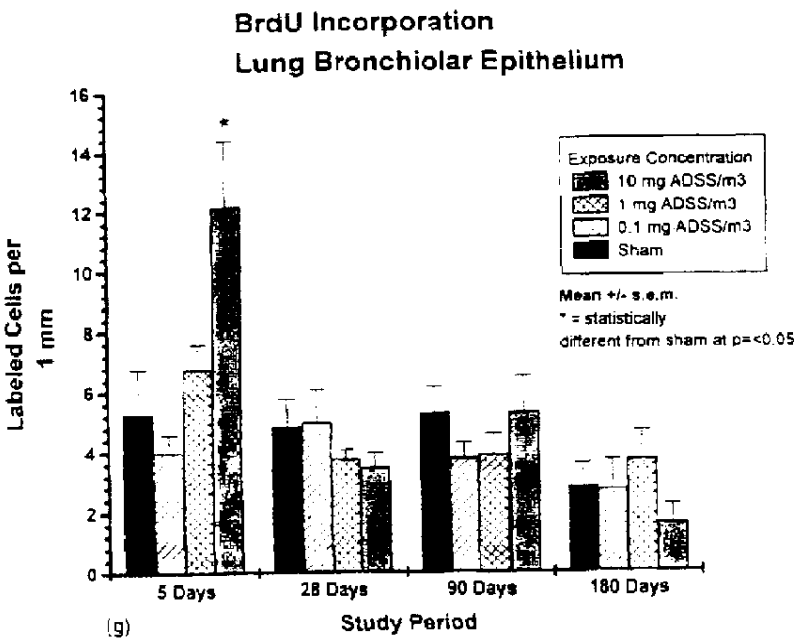


FIGURE 2. (*Continued*) Bar charts of the number of BrdU-labeled cells at study days 5, 28, 90, and 180: (g) in the lung bronchiolar epithelium.

In the nasal section 1 cuboidal epithelium of the high exposure group, cell labeling was significantly increased, about fivefold higher than the sham-exposed group (Figure 2a).

The respiratory epithelium of nasal section 1 was statistically increased in the high exposure group with about a twofold increase in cell labeling when compared to sham-exposed controls (Figure 2c). No other significant findings were noted at any exposure concentration at day 28.

Day 90

Statistically significant findings were noted only in the nasal cavity in the most rostral nasal section 1. There were no other statistically significant findings observed.

In the initial scoring of labeled cells in the nasal section 1 cuboidal epithelium, with knowledge of treatment group, statistically significant findings were noted at the low, mid, and high exposure groups when compared to the sham control. Cell labeling in the low and mid exposure groups were increased about twofold when compared to the sham exposed group. In the high exposure group, cell labeling was increased about sevenfold over that of the sham-exposed group.

Cell labeling was statistically increased in the respiratory epithelium of nasal section 1 at the high exposure group. Cell labeling in the respiratory

epithelium of nasal section 1 of the high exposure group was increased about twofold higher than the sham group (Figure 2c).

Day 90, Second Scoring of Nasal Section 1, Cuboidal Epithelium

BrdU-labeled cells of the nasal section 1 cuboidal epithelium at the 90-day time point were rescored, without knowledge of the treatment group. The rationale for including a second reading of this site at the 90-day assessment is provided in the discussion section.

Both sides of the nasal cavity at the nasal section 1 cuboidal epithelium site were counted separately and statistically compared at the 90 day period. Because no statistical difference was noted in the comparison of both sides of the nasal cavity, the cell count data were summed and averaged in this second scoring of nasal section 1 cuboidal epithelium at 90 days, in essence doubling the number of cells counted from the sensitive region. The data from both the initial scoring with knowledge of treatment group and the second scoring without knowledge of treatment are presented in Table 3.

In the rescoring of the nasal section 1 cuboidal epithelium site at 90 days, only the high exposure group was found to be significantly increased when compared to the sham exposed control. Cell labeling in the high exposure group was increased about sixfold over that of the sham control, whereas the number of labeled cells in the low and mid exposure groups that were not significantly different from the sham-exposed group were 1.53 and 1.51 times greater than the sham group, respectively (Figure 2a).

Day 180

Cell labeling after a 13-wk recovery period without smoke exposure was not statistically different from the sham-exposed group at any exposure concentration.

DISCUSSION

Exposure of male rats for 6 h/day in a subchronic study to aged and diluted sidestream smoke (ADSS) from reference cigarettes at concentrations of 1 or 10 mg/m^3 over a 5-, 28-, or 90-day period resulted in statistically significant increases in BrdU incorporation in some tissues when compared to sham-exposed animals. At the end of the 90-day exposure period, effects were only observed at the high concentration at the most rostral portion of the nasal cavity. The diminution of effects with increasing time on study suggests that adaptation to ADSS exposure was observed over the course of the study. Additionally, effects observed during exposure of rats to ADSS were not sustained after cessation of exposure.

Two evaluations of the incorporation of BrdU were conducted in the nasal section 1 cuboidal epithelium site at the 90-day assessment. The second evaluation was conducted for several reasons. The data at the low and

mid exposure concentrations, at 90 days, lacked any dose-response relationship, but both the low and mid concentrations were observed to be statistically different from the sham-exposed animals. If the response observed at the low concentration was related to exposure to ADSS, then a dose-related increase in BrdU incorporation at the mid dose should have been observed. No such increase was observed in the original evaluation of the slides.

The nasal section 1 cuboidal epithelium site exposed to the mid concentration of ADSS at 5 days incorporated statistically more BrdU than sham-exposed animals at 5 days. At this same site after 28 days, the incorporation rate of BrdU was no longer different. This observation suggested that this tissue site had adapted to the exposure of ADSS at the mid concentration. A similar adaptation was observed in four additional sites at which statistically significant increases in BrdU incorporation at the highest exposure concentration were observed at day 5 but were absent at days 28 and 90. The observation of statistically significant responses at the nasal section 1 cuboidal epithelium site in both the low and mid concentrations at 90 days was not consistent with the adaptation responses observed for this and other tissues at the 28- and 90-day evaluations relative to the 5-day observations. This inconsistency of the response at this tissue site at the 90-day assessment was apparent in the original evaluation of the data.

The magnitude of the statistically significant responses, at the low and mid concentrations, observed at the nasal section 1 cuboidal epithelium site, exceeded the sham control responses by a factor of two. Because the responses were only minimally different and the variability of the data on the order of 30–100%, only limited confidence could be placed in these observations specifically. A more rigorous assessment of the finding at the nasal section 1 cuboidal epithelium site was deemed necessary, based on the inconsistency of the apparent adaptation response, the lack of expected dose-response, and the minimal differences in the magnitude of the low and mid exposure effects. For this assessment, a doubling of the number of cells in the sensitive region of this site was evaluated. Additionally, the evaluation was conducted without prior knowledge of treatment. This approach was included to eliminate any potential for unintentional bias. The results of this more in-depth assessment supercede the initial findings from the reading of nasal section 1 cuboidal epithelium conducted with knowledge of treatment. All conclusions are based on this final interpretation of the BrdU-labeled cell counts.

The effects of ADSS exposure were not additive over the duration of the study. At the 5-day time point, statistically significant increases in BrdU incorporation were observed at 3 anatomical sites with responses observed at the mid and high exposure concentrations. The findings at 5 days were primarily limited to the nasal cavity along with a single finding in the lung

bronchiolar epithelium at the high concentration, which was not observed at any other time point in the study. At the 28-day time point, statistically significant increases in BrdU incorporation were observed at only 2 sites of the nasal cavity and only at the high exposure concentration. At the 90-day time point, the only sites that were statistically different from the sham controls were the nasal section 1 respiratory and cuboidal epithelium at the highest exposure concentration. Since, at the end of the 90-day exposure period, effects were observed at a lower number of sites and only at the high exposure concentration, in contrast to an additive phenomenon, these findings suggest that adaptation to the potential effects of ADSS exposure had occurred.

The statistical findings at the olfactory section 2 epithelium at the 5-day period are not biologically plausible. In the nasal section 2 olfactory epithelium at 5 days, the region termed olfactory site 1 was statistically different from the sham-exposed group at the high concentration while the olfactory site 2 was statistically different from the sham-exposed group only at the low concentration. Yet the only possible difference in the two sites is that site 1 was scored on one side of the nasal cavity and site 2 was scored on the other side of the nasal cavity in the same anatomical region. However, since no effort was made to maintain a left or right side orientation during slide preparation, and since these animals were exposed in the same manner for the same duration, differential responses at these two sites are not plausible. This discordance in the data suggests that these findings were spurious and of no biological significance.

Observation of a reduction in cell labeling in the sham-exposed animals with time on study confounded and therefore limited comparison of exposure groups across time points. The decrease in cell labeling as animals age on study has been observed by other investigators (Goldsworthy et al., 1991).

The scientific literature contains much information concerning the usefulness of replicative DNA synthesis data in interpretation of chronic effects from potentially toxic agents. Many of the compounds that induce increased rates of replicative DNA synthesis and have reported tumorigenic effects do so only at the maximum tolerated dose when increased cell killing, mitogenesis, and increased rates of mutagenesis occur (Ames & Gold, 1990). When the rate of replicative DNA synthesis exceeds the rate of cell death, mathematical models suggest that sustained cell proliferation may play a role in tumor development (Moolgavkar & Luebeck, 1991; Chen & Farland, 1991; Cohen & Ellwein, 1990). Experimental data both support and reject this hypothesis. In a study of replicative DNA synthesis in rats treated with the peroxisome proliferator Wy-14,643, a positive correlation was found between the rate of replicative DNA synthesis, which was sustained at about 5- to 10-fold higher than controls, and hepatocarcinogenesis (Marsman et al., 1988). In an acute study of BrdU incorporation into replicating hepatic cells, 2,4-diamino-

toluene induced replicative DNA synthesis that was correlated with the tumorigenic effect of 2,4-diaminotoluene in a chronic bioassay (Cunningham et al., 1991).

However, increased rates of replicative DNA synthesis sometimes are inversely correlated with tumor formation. In a study of 1,4-dichlorobenzene, hepatocellular tumors were induced in mice but not in rats. When liver cell replicative DNA synthesis rates were evaluated in both species treated with 1,4-dichlorobenzene, increased rates of replicative DNA synthesis were noted in both mice and rats (Eldridge et al., 1991). In an acute inhalation study investigating the cell proliferative effects of the nasal irritants formaldehyde and acrolein, both formaldehyde and acrolein demonstrated an increase in replicative DNA synthesis response acutely, even though a carcinogenic effect after chronic exposure is only seen with formaldehyde (Roemer et al., 1993). A recent study has reported that the predictive potential of increased replicative DNA synthesis in terms of individual animal cancer risk is weak. In a mouse skin tumor model, neither increased DNA adduct levels in skin nor increased replicative DNA synthesis was predictive of the individual cancer risk in the animals tested (Fischer et al., 1993). According to a recent report that included information about 19 chemicals tested by inhalation exposure in rodents, the association between proliferative changes such as increased replicative DNA synthesis and hyperplasia in nasal tissues and nasal carcinogenesis is weak. Of the 19 compounds tested, 5 were reported to be nasal carcinogens in the rodent. All five of the rodent nasal carcinogens displayed proliferative changes in the nose. Of the 14 compounds tested that were not reported to be nasal carcinogens, 12 demonstrated similar proliferative changes in the nose as did the nasal carcinogens, suggesting that proliferative changes alone are not predictors of nasal carcinogenesis (Ward et al., 1993). Therefore, the data reported here cannot be used to support the hypothesis that ADSS is a potential nasal carcinogen in the rat.

In this study, lack of statistically significant findings at the end of the recovery period after a 13-wk period without smoke exposure indicates that the effects observed at the end of 90 days were not sustained after cessation of exposure. The diminution of effects over the course of the 13-wk exposure period indicates that the animals adapted to the potential effects of ADSS exposure. The statistically significant findings in the nasal section 1 coincide with the histopathological effects noted in the nasal section 1 reported earlier (Coggins et al., 1993). In general, statistically significant findings were noted only in nasal tissues of the rat after subchronic exposure to the highest exposure concentration of ADSS, which was approximately 200- to 400-fold higher than reported concentrations of environmental tobacco smoke (Guerin et al., 1992). Considered with other data gathered by this laboratory (Coggins et al., 1993), these data of replicative DNA synthesis indicate that at the termination of a subchronic inhalation study, the no-observed-effect level (NOEL) for ADSS is greater than 1 mg ADSS/m^3.

REFERENCES

Ames, B. N., and Gold, L. S. 1990. Chemical carcinogenesis: Too many rodent carcinogens. *Proc. Natl. Acad. Sci. USA* 87:7772–7776.

Ayres, P. H., Mosberg, A. T., and Coggins, C. R. E. 1994. Design, construction, and evaluation of an inhalation system for exposing experimental animals to environmental tobacco smoke. *Am. Ind. Hyg. Assoc. J.* 55:806–810.

Butterworth, B. E. 1991. Chemically induced cell proliferation as a predictive assay for potential carcinogenicity. In Chemically induced cell proliferation: Implications for risk assessment. *Prog. Clin. Biol. Res.* 369:457–467.

Chen, C., and Farland, W. 1991. Incorporating cell proliferation in quantitative cancer risk assessment: Approaches, issues, and uncertainties. In Chemically induced cell proliferation: Implications for risk assessment. *Prog. Clin. Biol. Res.* 369:481–499.

Coggins, C. R. E., Ayres, P. H., Mosberg, A. T., Ogden, M. W., Hayes, A. W., and Sagartz, J. W. 1992. Fourteen-day inhalation study in rats, using aged and diluted sidestream smoke from a reference cigarette. I. Inhalation toxicology; histopathology. *Fundam. Appl. Toxicol.* 19:133–140.

Coggins, C. R. E., Ayres, P. H., Mosberg, A. T., Sagartz, J. W., and Hayes, A. W. 1993. Subchronic inhalation study in rats using aged and diluted sidestream smoke from a reference cigarette. *Inhal. Toxicol.* 5:77–96.

Cohen, S. M., and Ellwein, L. B. 1990. Cell proliferation in carcinogenesis. *Science* 249:1007–1011.

Clayson, D. B., Nera, E. A., and Lok, E. 1989. The potential for the use of cell proliferation studies in carcinogen risk assessment. *Regul. Toxicol. Pharmacol.* 9:284–295.

Cunningham, M. L., Foley, J., Maronpot, R. R., and Matthews, H. B. 1991. Correlation of hepatocellular proliferation with hepatocarcinogenicity induced by the mutagenic noncarcinogen:carcinogen pair 2,6- and 2,4-diaminotoluene. *Toxicol. Appl. Pharmacol.* 107:562–567.

Doolittle, D. J., McKarns, S. C., Ayres, P. H., and Bombick, D. W. 1992. Molecular approaches for quantifying DNA synthesis and cell proliferation during rodent bioassays. *Toxicol. Methods* 1:215–230.

Eldridge, S. R., Goldsworthy, T. L., Popp, J. A., and Butterworth, B. E. 1992. Mitogenic stimulation of hepatocellular proliferation in rodents following 1,4-dichlorobenzene administration. *Carcinogenesis* 13:409–415.

Feldman, D. B., and Seely, J. C. 1988. *Necropsy guide: Rodents and the rabbit*, pp. 1–50. Boca Raton, Fla.: CRC Press.

Fischer, W. H., Beland, P. E., and Lutz, W. K. 1993. DNA adducts, cell proliferation and papilloma latency time in mouse skin after repeated dermal application of DMBA and TPA. *Carcinogenesis* 14:1285–1288.

Goldsworthy, T. L., Morgan, K. T., Popp, J. A., and Butterworth, B. E. 1991. Guidelines for measuring chemically induced cell proliferation in specific rodent target organs. In Chemically induced cell proliferation: Implications for risk assessment. *Prog. Clin. Biol. Res.* 369:253–284.

Guerin, M. R., Jenkins, R. A., and Tomkins, B. A. 1992. *The chemistry of environmental tobacco smoke: Composition and measurement*. Chelsea, MI: Lewis.

Lumb, W. V., and Jones, E. W. 1984. Anesthesia of laboratory and zoo animals. In *Veterinary anesthesia*, eds. W. V. Lumb and E. W. Jones, pp. 413–491. Philadelphia: Lea and Febiger.

Marsman, D. S., Cattley, R. C., Conway, J. G., and Popp, J. A. 1988. Relationship of hepatic peroxisome proliferation and replicative DNA synthesis to the hepatocarcinogenicity of the peroxisome proliferators di(2-ethylhexyl)phthalate and [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid (Wy-14,643) in rats. *Cancer Res.* 48:5739–6744.

Monticello, T. M., Morgan, K. T., and Hurtt, M. E. 1990. Unit length as the denominator for quantitation of cell proliferation in nasal epithelia. *Toxicol. Pathol.* 18:24–31.

Moolgavkar, S. H., and Luebeck, E. G. 1991. The role of somatic mutations and cell replication kinetics in quantitative cancer risk assessment. In Chemically induced cell proliferation: Implications for risk assessment. *Prog. Clin. Biol. Res.* 369:469–479.

Roemer, E., Anton, H. J., and Kindt, R. 1993. Cell proliferation in the respiratory tract of the rat after acute inhalation of formaldehyde or acrolein. *J. Appl. Toxicol.* 13:103–107.

Sagartz, J. W., Madarasz, A. J., Forsell, M. A., Burger, G. T., Ayres, P. H., and Coggins, C. R. E. 1992.

Histological sectioning of the rodent larynx for inhalation toxicity testing. *Toxicol. Pathol.* 20:118–121.

von Meyerinck, L., Scherer, G., Adlkofer, F., Wenzel-Hartung, R., Brune, H., and Thomas, C. 1989. Exposure of rats and hamsters to sidestream smoke from cigarettes in a subchronic inhalation study. *Exp. Pathol.* 37:186–189.

Ward, J. M., Uno, H., Kurata, Y., Weghorst, C. M., and Jang, J. 1993. Cell proliferation not associated with carcinogenesis in rodents and humans. *Environ. Health Perspect.* 101(5):125–136.

Young, J. T. 1981. Histopathologic examination of the rat nasal cavity. *Fundam. Appl. Toxicol.* 1:309–312.